



UNIVERSITÀ
DEGLI STUDI
FIRENZE

FLORE

Repository istituzionale dell'Università degli Studi di Firenze

Simultaneous determination of anthocyanins, coumarins and phenolic acids in fruits, kernels and liqueur of *Prunus mahaleb* L

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

Original Citation:

Simultaneous determination of anthocyanins, coumarins and phenolic acids in fruits, kernels and liqueur of *Prunus mahaleb* L / F. Ieri; P. Pinelli; A. Romani. - In: FOOD CHEMISTRY. - ISSN 0308-8146. - STAMPA. - 135:(2012), pp. 2157-2162. [10.1016/j.foodchem.2012.07.083]

Availability:

This version is available at: 2158/775163 since: 2016-01-27T12:06:20Z

Published version:

DOI: 10.1016/j.foodchem.2012.07.083

Terms of use:

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

Publisher copyright claim:

(Article begins on next page)



Analytical Methods

Simultaneous determination of anthocyanins, coumarins and phenolic acids in fruits, kernels and liqueur of *Prunus mahaleb* L

Francesca Ieri*, Patrizia Pinelli, Annalisa Romani

Department of Pharmaceutical Sciences, University of Florence, via Ugo Schiff 6, 50019 Sesto Fiorentino (FI), Italy

ARTICLE INFO

Article history:

Received 1 July 2011

Received in revised form 12 March 2012

Accepted 16 July 2012

Available online 24 July 2012

Keywords:

White mahlab

HPLC/DAD/MS

Anthocyanins

o-Coumaric acid glucoside

Coumarin

Mirinello liqueur

ABSTRACT

In the fresh tissues of *Prunus mahaleb* L., three classes of phenolics were characterised: phenolic acid derivatives (main compound being o-coumaric acid glucoside), quercetin glycosides, and anthocyanins (cyanidin 3,5-diglucoside, cyanidin 3-sambubioside, cyanidin 3-xylosyl-rutinoside and cyanidin 3-rutinoside). Coumarin was also identified. The kernels showed a high content of coumarin (0.87 mg g^{-1}) which is the main class of metabolites in this sample, but present in pitted berries as well (0.63 mg g^{-1}). Flavonoids are mainly concentrated in the skin and pulp (0.55 mg g^{-1}). In 'Mirinello di Torremaggiore' liqueur, produced from *P. mahaleb* L. in accordance with traditional procedures, anthocyanins make up 16.5%, phenolic acids 43.3%, coumarin 36.2% and flavonoids 4% of total compounds. Anthocyanins are the main class in solid residues from liqueur production (70%). These findings point out that solid residues of *P. mahaleb* can be considered an interesting and innovative source of appreciable amounts of cyanidin glycosides (3.3 mg g^{-1}).

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

White mahlab (*Prunus mahaleb* L.) known also as English cherry, Rock cherry, St. Lucie cherry, of the *Rosaceae* family, subfamily *Prunoidae*, is a deciduous tree, 1–2 m high. Mahaleb cherry grows abundantly in West Asia; it is, however, sometimes found in Eastern and Central Europe, where it prefers a warm and dry climate. Mahaleb cherry trees, being rather robust and resistant to diseases, are commonly used as stock for grafting cherries, especially in the USA (Aydin, Ögüt, & Konak, 2002).

The kernels offer an important source of protein (30.98%) and oil (40.40%). Its oil is valuable in the preparation of lacquers and varnishes; it contains few cyanogenic glycosides and coumarin derivatives (Aydin et al., 2002). In Sudan the kernels are used for flavouring in bread, as cosmetic for wedding preparation and as a remedy for diarrhoea in children.

The leaves and bark of a wide range of *Prunus* species have been surveyed for the presence of coumarins. In particular, coumarin and herniarin (7-methoxycoumarin) were found in leaves, fruits and in the wood of roots of *P. mahaleb* (Favre-Bonvin, Massias, Mentzer, & Massicot, 1968; Santamour & Riedel, 1994).

Coumarin is a natural product well known for its pleasant vanilla-like odour. It has been reported in many plants belonging to a variety of families, including *Fabaceae*, i.e. Tonka bean (*Coumarouna odorata*) (Ehlers, Pfister, Bork, & Toffel-Nadolny, 1995) or

sweetclover (*Melilotus alba*) (Akeson, Gorz, & Haskins, 1963); *Lamiaceae*, i.e. lavender (*Lavandula officinalis*) (Brown, 1962); and *Lauraceae*, i.e. cinnamon (*Cinnamomum verum*) (Miller, Poole, & Pawlowski, 1996). There have been many reports on the effect of coumarin in plants, at the organ, tissue and cellular levels (Brown, 1981). These observations tend to demonstrate that coumarin acts as a plant hormone. However, until now, neither solid evidence for a physiological function nor the molecular mode of action of coumarin has been illustrated in plant tissues.

These molecules are found in higher plants where they originate from the general phenylpropanoid pathway (Harborne, 1999) and are subject to numerous modifications. Coumarins continue to receive attention for their diverse bioactivities. Some natural coumarins have been used as human therapeutics, while 4-hydroxycoumarins are prominent examples of a microbial modification which gave rise to the first generation molecules developed along with aspirin and heparin as anticoagulants (Mueller, 2004).

Coumarin was first suspected to have genotoxic and carcinogenic effects in the 1980s (AFC, 2004). On this basis, the Codex alimentarius outlined general requirements for natural flavourings, including specific maximum levels for coumarin in final ready-for-consumption products (Codex alimentarius, 1987). For foods and beverages in general, the maximum level is 2 mg kg^{-1} , except for special caramels and alcoholic beverages for which the maximum level is 10 mg kg^{-1} (Codex alimentarius. General requirements for natural flavourings (CAC/GL 29., 1987). Based on the non-observed-adverse-effect level (NOAEL) for hepatotoxicity in

* Corresponding author. Tel.: +39 0554573676.

E-mail address: francesca.ieri@unifi.it (F. Ieri).

animal experiments, the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) established a tolerable daily intake (TDI) of 0.1 mg kg⁻¹ bw (body weight) (AFC, 2004). The HPLC–DAD analysis of black and white mahlab seedcake extracts revealed the presence of phenolic compounds: (+)-catechin, chlorogenic, hydroxybenzoic, *p*-coumaric and syringic acids were detected. The presence of antioxidants in mahlab phenolic-rich fractions reduced the oxidation of β -carotene by hydroperoxides (Mariod, Ibrahim, Ismail, & Ismail, 2010).

White mahlab berries have a dark red colour, and among all common fruits and vegetables in the human diet, berries with dark blue or red colours have the highest antioxidant capacities (Wu et al., 2004).

The interest in anthocyanins, whose main qualities are an attractive bright colour, water solubility, and their easy incorporation into aqueous systems, has recently increased due to their beneficial health effects (Wang, Cao, & Prior, 1997; Zafra-Stone et al., 2007). Major phenolics in sweet cherries are anthocyanins, especially in dark-coloured sweet cherries (Gao & Mazza, 1995; Macheix, Fleuriet, & Billot, 1990). Dark-coloured genotypes have been found to contain 3-rutinoside and 3-glucoside of cyanidin as major anthocyanins and the same glycosides of peonidin as minor anthocyanins (Esti, Cinquanta, Sinesio, Moneta, & Di Matteo, 2002; Gao & Mazza, 1995; Macheix et al., 1990).

The aim of this paper was the identification and quantification of the secondary metabolites in white mahlab fruits, kernels and liqueur of *P. mahaleb* L., an indigenous crop of Puglia region (Italy), by combining data obtained by diode array detection (DAD) and electrospray ionisation mass spectrometry (ESI-MS). A single exhaustive extraction for all the classes of compounds and a single analytical HPLC method are proposed. A further objective of this study is to create a novel opportunity for using *P. mahaleb* L. for innovative food application (as food supplement) and for employing the waste materials as new source of natural pigments for food industry.

2. Materials and methods

2.1. Samples

Berries were collected from natural populations in Torremaggiore, Puglia (Italy). Intact berries, skin with flesh (pitted berries) and kernels were considered. A typical liqueur 'Mirinello di Torremaggiore' – MÈRÈNELLÈ of Italian tradition (19th century) was also analysed (Executive Decree, 10/07/06, Ministero delle politiche agricole, alimentari e forestali). For liqueur preparation, 0.5 kg of intact fruits were extracted with 1 L of 95% ethanol in infusion for 40 days and then the residues were filtered. The residues (peel and kernels) which remained after obtaining the liqueur were also considered.

2.2. Chemical reagents and materials

Acetonitrile was provided by E. Merck (Darmstadt, Germany), water was purified with a milli-Q system. Authentic standards of quercetin 3-glucoside ($\geq 99\%$ HPLC), keracyanin chloride ($\geq 96\%$ HPLC), trans-cinnamic acid ($\geq 95\%$ HPLC) and coumarin ($\geq 99\%$ GC) were purchased from Extrasynthèse (Genay Cedex-France).

2.3. Extraction of polyphenols

A 5 g sample of whole berries, pitted berries and kernels was crushed and then extracted with 50 mL of 70% ethanol, adjusted to pH 2.1 with hydrochloric acid to avoid degradation of anthocyanins, for one night at room temperature. The liqueur was analysed

as such. The residues (peel and kernels) which remained after obtaining the liqueur were treated as reported for the berries. All the samples were analysed in triplicate and the standard deviation was $<5\%$.

2.4. Analytical techniques and equipment

2.4.1. HPLC/DAD/MS analysis

Analysis was carried out using a HP-1100 liquid chromatograph equipped with a DAD detector and a HP 1100 MSD API-electrospray (Agilent-Technologies, Palo Alto, USA). Positive ionisation mode was applied to detect the anthocyanins and coumarins and the negative mode for the phenolic acids; different fragmentors, in the range 80–150 V, were applied. The column was a Synergi Max RP 80 A (4 μ m; 150 \times 3 mm i.d.) from Phenomenex. The mobile phase was (A) water pH 2.0 acidified by formic acid and (B) acetonitrile. The following multi-step linear gradient was applied: from 95% to 85% of A in 8 min, 12 min to reach 75% A, then 10 min to arrive at 5% A, which was maintained for 4 min. Total time of analysis was 34 min, flow rate 0.4 mL/min and oven temperature 26 \pm 0.5 °C.

UV–vis spectra were recorded in the 190–600 nm range and the chromatograms were acquired at 254, 280, 330, 350 and 520 nm.

2.4.2. HPLC/MS/MS Analysis

HPLC-MS/MS analyses were performed using a liquid chromatograph HP 1100 L interfaced with an API3000 mass spectrometer with triple quadrupole (Applied Biosystem-Sciex, Toronto, Canada) equipped with a turbo ion spray (TIS). The mass spectrometer operated at 5500 V of energy potential, turbo gas flow 7 L min⁻¹, and $T = 350$ °C. Collision-activated dissociation (CAD) MS/MS was performed in a LINAC Q2 collision device, using N₂ at 7 mTorr as collision gas. Declustering potential (DP) and collision energy (CE) were automatically optimised for each molecule by the software Analyst 1.4. MS, and MS/MS spectra were registered in continuous flow, connecting the infusion pump directly at the turbo ion spray source. Data were processed by the software Analyst 1.4 with the "Explore" option for spectra identification.

2.5. Identification and quantification of individual polyphenols

The identity of polyphenols was ascertained using data from HPLC/DAD, HPLC/MS and HPLC/MS/MS analyses, and by comparison and combination of their retention times, UV–vis and mass spectra with those of authentic standards. Quantification of individual polyphenolic compounds was directly performed by HPLC/DAD using a five-point regression curve ($r^2 \geq 0.998$) on the basis of authentic standards. In particular, flavonols such as quercetin derivatives were determined at 350 nm using quercetin 3-O-glucoside as reference compound. Anthocyanins were determined at 520 nm using cyanidin-3-O-rutinoside chloride (keracyanin) as reference compound while phenolic acids and coumarins were determined at 280 nm using cinnamic acid and coumarin respectively as reference compounds. In all cases, actual concentrations of the derivatives were calculated after applying corrections for differences in molecular weight, in particular knowing the molecular weight of each compound (PM_x) it is possible to obtain its actual concentration by applying a multiplication factor of PM_x/PM_y, where PM_y is the molecular weight of the specific reference compound (Amitabh, Jatinder, & Yingqin, 2001).

3. Results and discussions

The separation of white mahlab metabolites was performed in reversed-phase high performance liquid chromatography (RP-

HPLC). To identify and classify the phenolic compounds, the UV–visible absorption spectra acquired with diode array detector (DAD) was used. DAD is currently the most widely available and commonly used technique for routine qualitative and quantitative analysis of these metabolites (He, 2000; Merken & Beecher, 2000).

The combination of these data with mass spectra (MS) data and information from the respective literature or from comparison with standard compounds can be used for tentative identification of each peak in a chromatogram (He, 2000; Robbins, 2003).

In the fresh tissues of white mahlab (i.e., flesh with skin and kernels), three classes of phenolics were characterised: phenolic acid derivatives (main compound being *o*-coumaric acid diglucoside); flavonols (quercetin glycosides); and anthocyanins (cyanidin glycosides). Coumarin (benzo-2-pyrone) was also identified.

A single procedure was applied to simultaneously determine the four classes of metabolites.

As an example, Fig. 1 reports the chromatographic profiles registered at 280 nm and Fig. 2 at 520 nm of the hydroalcoholic berry extracts. The identification of single metabolites of intact berries are summarized in Table 1.

3.1. Phenolic acid derivatives

Phenolic acids were identified both in the flesh and seeds of white mahlab. Three hydroxycinnamic acid derivatives were tentatively identified: *o*-coumaric acid diglucoside; dihydro-*o*-coumaric acid 2-*O*-glucoside; *o*-coumaric acid 2-*O*-glucoside. LC–MS and MS–MS were used in positive and negative ionisation modes in order to obtain more information on the structural features of the conjugated forms of phenolic compounds. UV–visible spectra identification was crucial to allow the assignment of peaks as *o*-coumaric acid derivatives.

The peak with Rt 9.5 presented a quasi molecular ion m/z 487 $[M-H]^-$ and fragments m/z 325 $[M-H-hexose]^-$, m/z 163 $[M-H-hexose-hexose]^-$ (Table 1), coinciding with the mass of two glucose moieties linked to coumaric acid. In addition, a fragment ion $[M-H-hexose-hexose-44]^-$ corresponding to the loss of the $-CO_2$ group (carboxylic function) was detected.

The peak with Rt 12.3 showed a quasi molecular ion m/z 327 $[M-H]^-$ and a fragment m/z 163 $[M-H-hexose]^-$. The fragment

ion $[M-H-hexose-44]^-$ corresponding to the loss of the $-CO_2$ group was also detected. Dihydro-*o*-coumaric acid 2-*O*-glucoside was tentatively identified by UV–vis spectrum and MS data.

The peak with Rt 13.4 presented similar coumarin-type UV–vis spectra and quasi molecular ion m/z 325 $[M-H]^-$ and fragments m/z 163 $[M-H-hexose]^-$ and m/z 119 $[M-H-hexose-CO_2]^-$ (Fig. 1, Table 1), coinciding with the mass of a hexose moiety linked to *o*-coumaric acid. The glycosylation of the hydroxyl group in *o*-coumaric acid caused a hypsochromic shift (from 322 to 312 nm) and the disappearance of the typical spectral feature of the aglycone (Fig. 1). Sugar esters cause bathochromic shifts, whereas *O*-glycosidic bonds cause hypsochromic shifts compared to their aglycons (Monagas, Suárez, Gómez-Cordovés, & Bartolomé, 2005; Määtä, Kamal-Eldin, & Törrönen, 2003). These data revealed the presence of *o*-coumaric acid 2-*O*-glucoside.

Under our experimental conditions, for some derivatives clear fragmentation did not occur; among them compounds 5 and 6, Rt 30.4 and 33.6 respectively, showed similar UV–vis spectra. In particular peak 6 showed the fragment m/z 271 $[M-H]^-$, that corresponds to naringenin quasi-molecular ion, but Rt and UV–vis spectra do not agree with the flavanone commercial standard.

3.2. Coumarins

MS analyses of coumarins were performed in positive ionisation mode. The identification of the peak with Rt 23.4 as coumarin was confirmed by the injection of commercial standard. In addition to the quasi molecular ion, a fragment ion at m/z 103, corresponding to $[M+H-44]^+$, was detected (Table 1). As previously, reported coumarin was found in fruits of *P. mahaleb* (Favre-Bonvin et al., 1968) as well as in seeds and liqueur, whereas the extract ion current (EIC) of herniarin (7-methoxycoumarin) in berries, kernels extracts and liqueur produced no results.

3.3. Anthocyanins

The anthocyanin profile was determined for the pigmented berries and the chemical structures were determined by HPLC/DAD/MS and HPLC/MS/MS analyses by the mass spectra in positive ionisation mode at different fragmentation energy, and by comparison

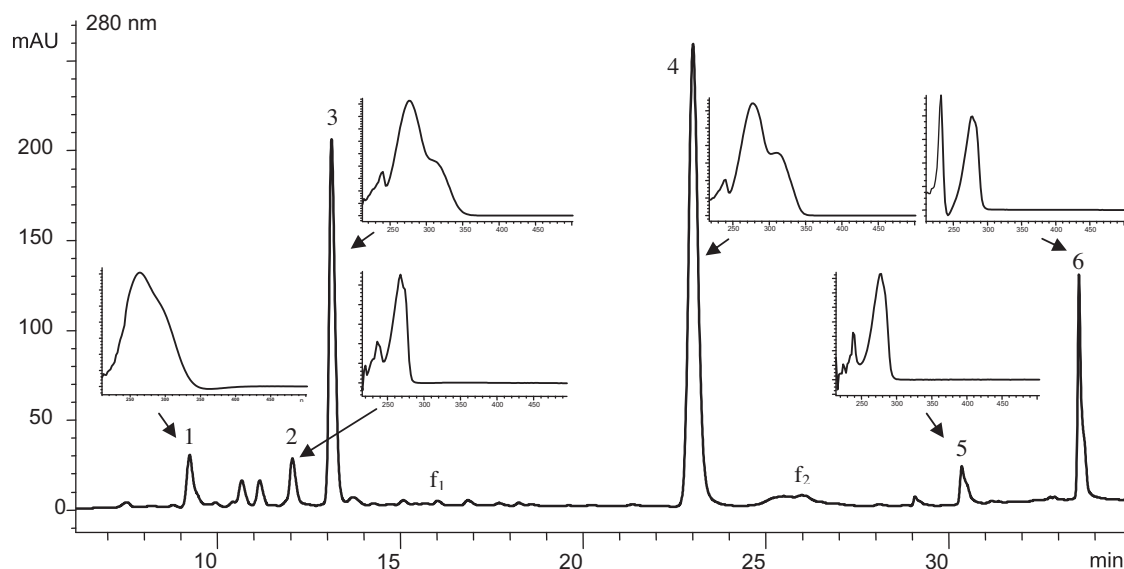


Fig. 1. Chromatographic profile at 280 nm of white mahlab berries hydroalcoholic extract. The following compounds were tentatively identified: (1) *o*-coumaric acid diglucoside; (2) dihydro-*o*-coumaric acid 2-*O*-glucoside; (3) *o*-coumaric acid 2-*O*-glucoside; (4) coumarin; (5) and (6) unknown compounds; (f₁) quercetin-3-glucoside; (f₂) quercetin derivative. Insets are UV–vis spectra of each compounds.

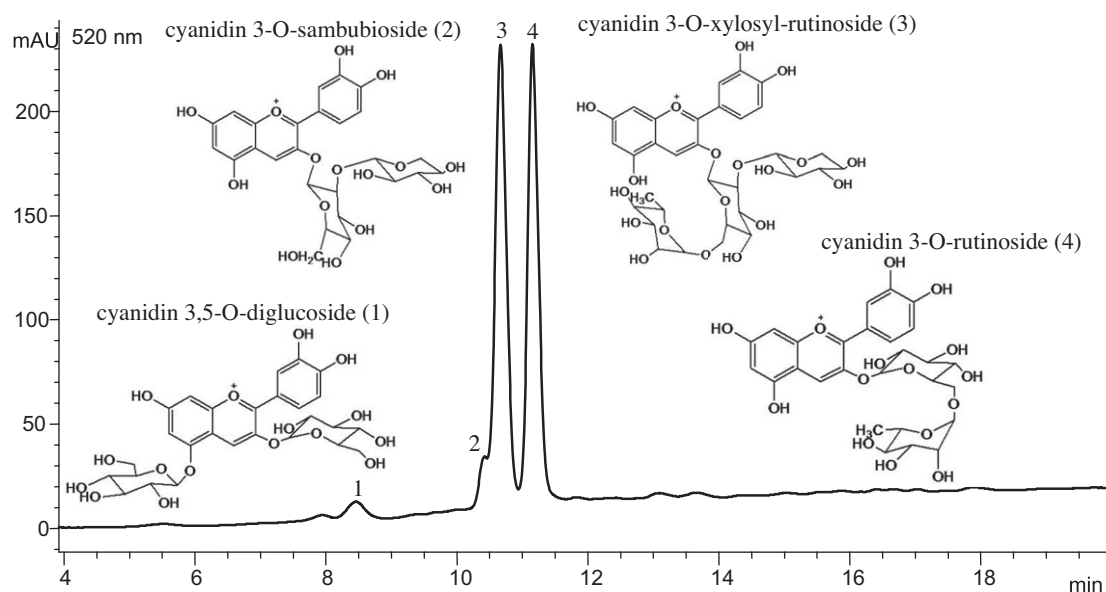


Fig. 2. Chromatographic profile at 520 nm of anthocyanins in white mahlab berries. The following molecules were identified: (1) cyanidin 3,5-O-diglucoside (m/z 611); (2) cyanidin 3-O-sambubioside (m/z 581); (3) cyanidin 3-O-xylosyl-rutinoside (m/z 727); (4) cyanidin 3-O-rutinoside (m/z 595).

Table 1

UV spectra and MS data obtained after positive and negative ionisation of the white mahlab hydroalcoholic extract of intact berries applying various fragmentation energies (from 80 to 100 V).

Tentative identification	Rt (min)	λ_{\max}	mw	Fragment ions (m/z)
Anthocyanins				
Cyanidin 3,5-O-diglucoside	8.4	516	611	<i>Ion mode 80 positive</i> 611, 449, 287
Cyanidin 3-O-sambubioside	10.4	516	581	581, 287
Cyanidin 3-O-xylosyl-rutinoside	10.7	516	727	727, 581, 287
Cyanidin 3-O-rutinoside	11.1	516	595	595, 449, 287
Phenolic acids and coumarins				
<i>o</i> -Coumaric acid diglucoside	9.5	264	488	<i>Ion mode 80–100 negative</i> 487, 325 (-gluc), 163 (-gluc), 119 (-CO ₂)
Dihydro- <i>o</i> -coumaric acid 2-O-glucoside	12.3	268	328	327, 165 (-gluc), 121 (-CO ₂)
<i>o</i> -Coumaric acid 2-O-glucoside	13.4	276, 312	326	325, 163 (-gluc), 119 (-CO ₂)
Coumarin	23.4	276, 312	146	147, 103 (-CO ₂)
Peak 5	30.4	278		
Peak 6	33.6	278		271
Flavonols				
Quercetin-3-glucoside	17.6	354	464	<i>Ion mode 80–100 negative</i> 463, 301
Quercetin derivative	27.3	370		301

Gluc = glucoside.

of their UV-vis spectra and Rt values with those of literature (Wu, Gu, Prior, & McKay, 2004).

The EIC profile at m/z 287 (cyanidin aglycone) showed four peaks with m/z 611, 581, 727 and 595 which corresponded to the molecular cations of cyanidin 3,5-diglucoside, cyanidin 3-sambubioside, 3-xylosyl-rutinoside, and cyanidin 3-rutinoside, respectively (Table 1).

MS/MS product-ion analysis of cyanidin 3,5-diglucoside produced the aglycone cation and a fragment ion at m/z 449 corresponding to 3-substituted or 5-substituted anthocyanin, indicating that either the C-3 or C-5 glucose substituent was fragmented. Cyanidin 3-sambubioside was fragmented only to the aglycone cation (m/z 287, cyanidin) illustrating that the glycosidic bond between xylose and glucose did not fragment during MS/MS.

Cyanidin 3-xylosyl-rutinoside produced fragment ions at m/z 287 and m/z 581 that showed the loss of a rhamnose substituent and confirmed the non-fragmentation between xylose and glucose.

Cyanidin 3-rutinoside showed the aglycone cation and a fragment at m/z 449 that corresponded to loss of the rhamnose

substituent, indicative of the fragmentation of the glycosidic bond between glucose and rhamnose.

The anthocyanin profile of white mahlab was similar to the profile of red currant, rather than a sweet cherry anthocyanin pattern, where 3-rutinoside and 3-glucoside of cyanidin, peonidin and pelargonidin are reported (Macheix et al., 1990; Wu, Beecher, et al., 2004; Wu & Prior, 2005; Wu et al., 2004).

3.4. Flavonols

HPLC/DAD/MS data indicate that all flavonols are derivatives of quercetin. In particular the identification of quercetin 3-glucoside was confirmed by the injection of commercial standard. MS analysis in negative mode produced the aglycone fragment (m/z 301) and a fragment ion at m/z 463 corresponding to 3-substituted glucoside (Table 1).

The compound with Rt 27.3 shows a signal at m/z 301 and presents a typical UV-vis spectrum, thus confirming the presence of a quercetin derivative.

3.5. Contents and relative distributions of the individual compounds

The previous procedure was then applied to evaluate the quantitative content of these secondary metabolites in berries, skin with flesh (pitted berries), kernels, liqueur and solid residues after liqueur production (peel and kernels) of *P. mahaleb*. The qualitative HPLC/DAD profiles were very similar for all the samples, with the exception of the kernel extract, where anthocyanins were absent. Quantitative data, expressed as mg g^{-1} of fresh samples, are reported in Fig. 3. In particular, kernels, 33% of whole berries, showed a high content of coumarin (0.87 mg g^{-1}) which is the main class of metabolites in this sample, but present in pitted berries as well (0.63 mg g^{-1}). Seeds showed a smaller content of flavonols and phenolic acids with respect to pitted berries. The data revealed that flavonoids are mainly concentrated in the skin and pulp, and that anthocyanins are the main class in solid residues (70%).

Anthocyanins present in plants play a role in self-protection against biotic and abiotic stress and can contribute to chemotaxonomic characterization (Ortega-Regules, Romero-Cascales, Lopez-Roca, Ros-Garcia, & Gomez-Plaza, 2006). In the last decade, great interest has been developed regarding the evaluation of the anthocyanin content in the human diet due to their potential health benefits (McGhie & Walton, 2007; Zafra-Stone et al., 2007). In addition to health considerations, there is interest in anthocyanins for their possible use as natural colourants. They are water soluble, which facilitates their incorporation into aqueous food systems (Markakis, 1992), and have been consumed for centuries without adverse effects. Residues of *P. mahaleb* from liqueur preparation could be a new potential, cost-saving source of these interesting pigments. Residue extracts allow specific applications due to the reported biological activities and uses for several new formulations in different application fields.

Phenolic acids are the main class of metabolites in intact and pitted berries. It is interesting to note the presence of *o*-coumaric acid derivatives, precursors of coumarin biosynthesis. *Cis-o*-coumaric acid glucoside lactonizes to coumarin, so it is conceivable that coumarin is found in *P. mahaleb* extracts. Phenolic acids have been reported to possess important biological and pharmacological properties and may have benefits for human health; in particular, *o*-coumaric acid ameliorate obesity induced by high-fat diet in rats (Hsu, Wu, Huang, & Yen, 2009).

Data obtained from 'Mirinello di Torremaggiore' liqueur are reported in Table 2. All the classes of identified metabolites are present, in particular anthocyanins make up 16.5%, phenolic acids 43.3%, coumarin 36.2% and flavonols 4% of total compounds.

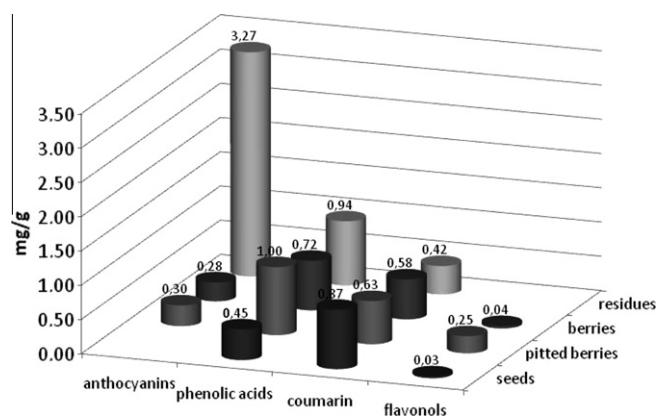


Fig. 3. Content of secondary metabolites in seeds, pitted berries (skin and flesh), berries (whole fruit) and residues. Data, expressed as mg g^{-1} of fresh sample, are a mean of three determination with a standard deviation <5%.

Table 2

Single phenolic compounds and coumarins quantification in 'Mirinello di Torremaggiore' liqueur.

Compound	Liqueur ($\text{mg L}^{-1} \pm \text{SD}$)
Cyanidin 3,5-O-diglucoside	22.37 \pm 1.0
Cyanidin 3-O-sambubioside	9.02 \pm 0.4
Cyanidin 3-O-xylosyl-rutinoside	168.56 \pm 8.3
Cyanidin 3-O-rutinoside	137.29 \pm 6.2
<i>o</i> -Coumaric acid diglucoside	n.d.
Dihydro- <i>o</i> -coumaric acid 2-O-glucoside	85.32 \pm 3.8
<i>o</i> -Coumaric acid 2-O-glucoside	760.15 \pm 36.5
Coumarin	740.26 \pm 36.3
Peak 5	2.10 \pm 0.1
Peak 6	37.10 \pm 1.7
Quercetin-3-glucoside	65.80 \pm 2.9
Quercetin derivative	16.10 \pm 0.7
Total metabolites	2044.07

Data are the mean \pm SD of three determinations and are expressed as mg L^{-1} .

The liqueur is produced in accordance with traditional procedures and uses and is consumed as a bitter digestive aid and remedy for digestive disorders. In order to ensure the beneficial effects of these natural ingredients, the packaging usually contains recommended amounts for use.

The experimental conditions applied in this work allowed determination of phenolic acids, anthocyanins and coumarins with sufficient resolution and sensitivity in a 34-min period. To recognise the 12 different compounds detected in *P. mahaleb* extracts, the use of a mass detector was crucial. Although all identified compounds are known chemically (Table 1), most of the metabolites have not been previously detected in berries, kernels extracts and liqueur of *P. mahaleb*.

To the best of our knowledge, this is the first report about the exhaustive phenols characterization of this species and its transformed product (liqueur). Moreover, the findings of the present study point out that solid residues of *P. mahaleb* from liqueur production can be considered an interesting sources of appreciable amounts of anthocyanins, in particular of cyanidin derivatives (3.3 mg g^{-1}).

Therefore, identification of anthocyanin-rich extracts could supply the demand by consumers and the food industry for natural pigments with added value, creating new opportunities for use of these extracts in a variety of applications.

Acknowledgments

We are grateful, to STIMA s.r.l. and BIOTECHNOLOGY Power s.r.l. for financial support, and to Ente Cassa di Risparmio di Firenze for supplying part of the instrumentation used in this research. In addition, technical support provided by Sandra Gallori is also kindly acknowledged.

References

- AFC. (2004). Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the Commission related to Coumarin. *EFSA Journal*, 104, 1–36.
- Akeson, W. R., Gorz, H. J., & Haskins, F. A. (1963). Effect of genotype and growth stage on distribution of mellilotic acid, *o*-coumaric acid, and coumarinic acid in *Melilotus alba* Desr. *Crop Science*, 3, 167–171.
- Amitabh, C., Jatinder, R., & Yingqin, L. (2001). Separation, identification, quantification, and method validation of anthocyanins in botanical supplement raw materials by HPLC and HPLC-MS. *Journal of Agricultural and Food Chemistry*, 49, 3515–3521.
- Aydin, C., Ögüt, H., & Konak, M. (2002). Some physical properties of Turkish Mahaleb. *Biosystems Engineering*, 82, 231–234.
- Brown, S. A. (1962). Biosynthesis of coumarin and herniarin in lavender. *Science*, 137, 977–978.
- Brown, S. A. (1981). Coumarins. In P. K. Stumpf & E. E. Conn (Eds.), *The biochemistry of plants – A comprehensive treatise* (pp. 269–300). New York: Academic Press.

- Codex alimentarius. General requirements for natural flavourings (CAC/GL 29.1987). www.codexalimentarius.net.
- Ehlers, D., Pfister, M., Bork, W. R., & Toffel-Nadolny, P. (1995). HPLC analysis of Tonka bean extracts. *Z Lebensm Unters Forsch*, 201, 278–282.
- Esti, M., Cinquanta, L., Sinesio, F., Moneta, E., & Di Matteo, M. (2002). Physicochemical and sensory fruit characteristics of two sweet cherry cultivars after cool storage. *Food Chemistry*, 76, 399–405.
- Favre-Bonvin, J., Massias, M., Mentzer, C., & Massicot, J. (1968). Sites de biosynthèse des coumarines chez *Prunus mahaleb*: Étude par greffage et administration d'un précurseur marqué. *Phytochemistry*, 7, 1555–1560.
- Gao, L., & Mazza, G. (1995). Characterization, quantitation, and distribution of anthocyanins and colorless phenolics in sweet cherries. *Journal of Agricultural and Food Chemistry*, 43, 343–346.
- Harborne, J. B. (1999). Classes and functions of secondary products from plants. In N. J. Walton & D. E. Brown (Eds.), *Chemicals from plants* (pp. 1–25). London: Imperial College Press.
- He, X. (2000). On-line identification of phytochemical constituents in botanical extracts by combined high-performance liquid chromatographic–diode array detection–mass spectrometric techniques. *Journal of Chromatography A*, 880, 203–232.
- Hsu, C. L., Wu, C. H., Huang, S. L., & Yen, G. C. (2009). Phenolic compounds rutin and *o*-coumaric acid ameliorate obesity induced by high-fat diet in rats. *Journal of Agricultural and Food Chemistry*, 57, 425–431.
- Määtä, K. R., Kamal-Eldin, A., & Törrönen, A. R. (2003). High-performance liquid chromatography (HPLC) analysis of phenolic compounds in berries with diode array and electrospray ionization mass spectrometric (MS) detection: *Ribes* species. *Journal of Agricultural and Food Chemistry*, 51, 6736–6744.
- Macheix, J. J., Fleuriot, A., & Billot, J. (1990). *Fruit phenolics*. Boca Raton, FL, USA: CRC Press.
- Mariod, A. A., Ibrahim, R. M., Ismail, M., & Ismail, N. (2010). Antioxidant activities of phenolic rich fractions (PRFs) obtained from black mahlab (*Monechma ciliatum*) and white mahlab (*Prunus mahaleb*) seedcakes. *Food Chemistry*, 118, 120–127.
- Markakis, P. (1992). Stability of anthocyanins in foods. In P. Markakis (Ed.), *Anthocyanins as food colors* (Chapter 6). New York: Academic Press.
- McGhie, T. K., & Walton, M. C. (2007). The bioavailability and absorption of anthocyanins: Towards a better understanding. *Molecular Nutrition & Food Research*, 51, 702–713.
- Merken, H. M., & Beecher, G. R. (2000). Measurement of food flavonoids by high-performance liquid chromatography: A review. *Journal of Agricultural and Food Chemistry*, 48, 577–599.
- Miller, K. G., Poole, C. F., & Pawlowski, T. M. P. (1996). Classification of the botanical origin of cinnamon by solidphase microextraction and gas chromatography. *Chromatographia*, 42, 639–646.
- Monagas, M., Suárez, R., Gómez-Cordovés, C., & Bartolomé, B. (2005). Simultaneous determination of nonanthocyanin phenolic compounds in red wines by HPLC–DAD/ESI-MS. *American Journal of Enology and Viticulture*, 56, 139–147.
- Mueller, R. L. (2004). First-generation agents: Aspirin, heparin and coumarins. *Best Practice & Research Clinical Haematology*, 17, 23–53.
- Ortega-Regules, A., Romero-Cascales, I., Lopez-Roca, J. M., Ros-Garcia, J. M., & Gomez-Plaza, E. (2006). Anthocyanin fingerprint of grapes: Environmental and genetic variations. *Journal of the Science of Food and Agriculture*, 86, 1460–1467.
- Robbins, R. J. (2003). Phenolic acids in foods: An overview of analytical methodology. *Journal of Agricultural and Food Chemistry*, 51, 2866–2887.
- Santamour, F. S., Jr., & Riedel, L. G. H. (1994). Distribution and inheritance of scopolin and herniarin in some *Prunus* species. *Biochemical Systematics and Ecology*, 22, 197–201.
- Wang, H., Cao, G., & Prior, R. L. (1997). Oxygen radical absorbing capacity of anthocyanins. *Journal of Agricultural and Food Chemistry*, 45, 304–309.
- Wu, X., & Prior, R. L. (2005). Systematic identification and characterization of anthocyanins by HPLC–ESI-MS/MS in common foods in the United States: Fruits and berries. *Journal of Agricultural and Food Chemistry*, 53, 2589–2599.
- Wu, X., Beecher, G., Holden, J., Haytowitz, D., Gebhardt, S. E., & Prior, R. L. (2004). Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *Journal of Agricultural and Food Chemistry*, 52, 4026–4037.
- Wu, X., Gu, L., Prior, R. L., & McKay, S. (2004). Characterization of anthocyanins and proanthocyanidins in some cultivars of *Ribes*, *Aronia*, and *Sambucus* and their antioxidant capacity. *Journal of Agricultural and Food Chemistry*, 52, 7846–7856.
- Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., Vinson, J. A., & Bagchi, D. (2007). Berry anthocyanins as novel antioxidants in human health and disease prevention. *Molecular Nutrition & Food Research*, 51, 675–683.